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STRUCTURAL HETEROGENEITY OF RECONSTITUTED COMPLEXES OF DNA WITH TYPICAL AND INTERMEDIATE PROTAMINES

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The binding of the intermediate proteins φ1 and φ3 from the mussel *Mytilus edulis* to DNA was studied in comparison with the typical protamine from the squid *Loligo vulgaris* using precipitation curves, thermal denaturation and X-ray diffraction techniques. The properties of protein φ1 appear to be very close to those of typical protamines while the properties of protein φ3 are notably different. The method of reconstitution influences the structural properties of the complexes. This effect is most pronounced in the case of protein φ3. The structural heterogeneity of the protein component in the complexes is discussed in the light of these observations.

1. Introduction

Proteins bound to DNA in sperm nuclei vary widely among different species. They were classified by Bloch [1] in five major classes on the basis of their composition: protamines (salmonids); stable protamines or basic keratins (mouse, grasshopper); intermediate protamines (mussel); histones (frog, carp); and nonbasic proteins (crab).

We have used precipitation curves, thermal denaturation and X-ray diffraction techniques to study some of the DNA-binding properties of the intermediate protamines φ1 and φ3 from the sperm of the mussel *Mytilus edulis* and compared them to those of the basic protein from the sperm of the squid *Loligo vulgaris* selected as a reference protamine.

Over two-thirds of the amino acid residues of squid protamine are arginine, which constitutes the sole basic amino acid (table 1). The X-ray diffraction of squid nucleoprotamine has been reported in detail elsewhere [2].

About one-half of the amino acid residues of proteins φ1 and φ3 are basic residues. Protein φ1 is the major basic component associated with DNA in mussel sperm nuclei and contains lysine and arginine in equivalent amounts. Protein φ3 is a minor basic component in nuclei [3,4]. It has a high content of lysine and a low content of arginine (table 1). On the basis of their composition proteins φ1 and φ3 have been included in the class of intermediate protamines.

Information on the interaction of intermediate protamines with DNA is very scanty. We show here how differences in composition between typical and intermediate protamines modify their DNA-binding properties. The structural aspects of this problem have been examined, determining the influence of the reconstitution methods on the structural heterogeneity of the protein component in the nucleoprotamine complexes.

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2. Materials and methods

2.1 Protein preparation

Sperm basic proteins from mussel (*M. edulis*) and squid (*L. vulgaris*) were obtained as described elsewhere [5]. Proteins from mussel were further purified by ion-exchange chromatography on CM-C25 Sephadex according to Willmitzer [6]. Purified proteins were examined on 15% acrylamide urea-acetic acid gel electrophoresis according to Panyim and Chalkley [7]. As shown in fig. 1, all proteins used in this study migrate as a single component under these electrophoretic conditions. Their amino acid composition is given in table 1.

2.2. Titration of DNA by sperm basic proteins

The complexes were prepared by direct binding in 10^{-3} M EDTA, pH 7.6, according to Weiskopf and Li [8]. Protein at a concentration of 0.2 mg/ml was added under continuous gentle stirring to a 10^{-4}M DNA solution. The complexes were centrifuged for 10 min at 12 000 *g*. The supernatants were measured for absorption at 260 and 320 nm. The (+/−) ratio was defined as Arg/nucleotide for squid protamine complexes and Lys + Arg/nucleotide for φ1 and φ3 complexes. It was calculated on the basis of the amino acid composition.

2.3. Thermal denaturation

Two different methods were used for the preparation of the complexes. (i) Rapid binding: protein and DNA were mixed in the buffer used for thermal denaturation by adding the protein to the DNA under gentle stirring; then, the complexes were extensively dialyzed against the buffer used for thermal denaturation (ii) Slow binding: protein and DNA were mixed in 2 M NaCl (protein φ1

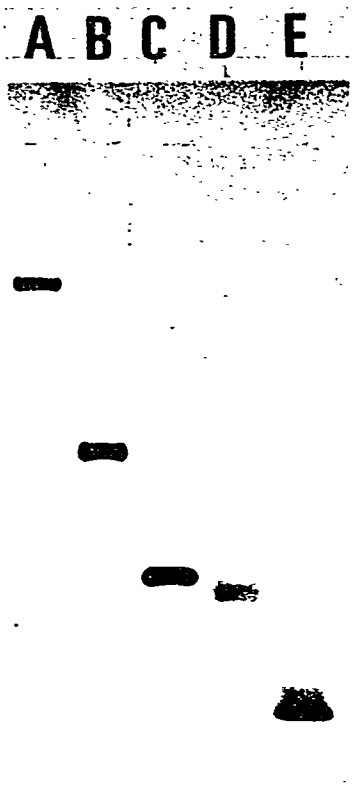

Fig. 1. Urea-acetic acid polyacrylamide gel electrophoresis of (a) calf thymus histone H1, (b) *M. edulis* φ1, (c) *L. vulgaris* protamine, (d) *M. edulis* φ3, (e) thynnin (tuna fish protamine). Histone H1 and thynnin are shown for comparison.

Table 1

Amino acid composition of the proteins used in this study

Species	Number of amino acid residues								Total number of residues	Refs.
	Lys	Arg	Thr	Ser	Pro	Gly	Ala	Tyr		
L. vulgaris protamine	–	55	–	9	2	–	–	5	71	5, 27
M. edulis φ1	22	29	4	17	6	6	14	–	99	4
M. edulis φ3	42	3	2	8	8	1	17	–	83	4

and $\phi 3$ complexes) or in 2 M guanidinium chloride (squid protamine complexes) and subjected to NaCl or guanidium chloride continuous exponential gradients down to 0.15 M; then the complexes were extensively dialyzed against the buffer for thermal denaturation.

Calf thymus DNA was used. It was sonicated to a molecular weight of approx. 10^5 ($s_w^{20} = 7$). Its concentrations were measured by ultraviolet absorption spectrophotometry using $A_{258} = 20$ cm^{-1} $(mg/ml)^{-1}$. The protein content of the solutions was measured with the Sakaguchi method as modified by Satake and Luck [9] in the case of squid protamine and with the trinitrobenzenesulfonate (TNBS) method [10] in the case of lysine-containing proteins $\phi 1$ and $\phi 3$.

The melting curves were obtained with a Beckman DU-2 spectrophotometer. Readings were taken every 2°C at a heating rate of 0.15°C/min. The ratio of the absorbances at 320 and 260 nm was taken as a measure of turbidity. The melting curves were normalized and plotted as hyperchromicity derivatives with respect to temperature, dh_{260}/dT, vs. temperature.

2.4. X-ray diffraction studies

X-ray diffraction patterns were recorded as described elsewhere [2]. For the preparation of the complexes the two methods described in section 2.3 were used. Chemical analysis of the fibers was accomplished as described elsewhere [2]. When the lysine-rich proteins from mussel were to be evaluated, the TNBS method [10] was used instead of Sakaguchi's method.

3. Results

3.1. Precipitation curves

Fig. 2 shows the precipitation curves for squid protamine and mussel $\phi 1$ and $\phi 3$. At low salt concentration (10^{-3} M EDTA, pH 7.4) squid protamine and $\phi 1$ precipitate DNA in a very narrow range of $(+/-)$ ratios, with the midpoint of the precipitation curve at $(+/-) = 0.98$. As in polylysine-DNA complexes precipitation takes place when almost all DNA phosphates are neutralized by protein basic residues. The precipitation of DNA at a narrow concentration range indicates that the binding of protamine on DNA is not highly cooperative.

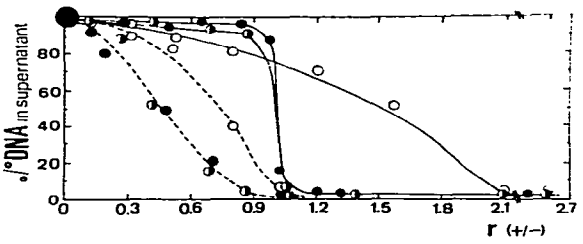

Fig. 2. Precipitation curves of DNA by *L. vulgaris* protamine (●), *M. edulis* $\phi 1$ (◑) and *M. edulis* $\phi 3$ (○). Titrations were carried out either in 10^{-3} M EDTA, pH 7.4 (continuous line), or in 0.15 M NaCl, 10^{-3} M EDTA, pH 7.4 (dotted line). The input ratio $(+/-)$ is reported as Arg/nucleotide for *L. vulgaris* protamine complexes or Arg + Lys/nucleotide for *M. edulis* $\phi 1$ and $\phi 3$ complexes.

Protein $\phi 3$ is very inefficient in precipitating the DNA at low ionic strength. A value of 2.1 for the $(+/-)$ ratio is necessary for complete precipitation of the DNA. Nevertheless, the precipitation of a small fraction of the DNA at low $(+/-)$ ratios suggests that some cooperativity and/or cross-linking of DNA molecules by protein $\phi 3$ could be present.

At 0.15 M NaCl the three proteins precipitate the DNA gradually. Complete precipitation is obtained at $(+/-) = 1$. The values of the $(+/-)$ ratio for 50% precipitation are approx. 0.5, approx. 0.5 and approx. 0.65 for squid protamine, protein $\phi 1$ and protein $\phi 3$, respectively. This behavior indicates that DNA molecules are saturated in turn by protamine. Thus, in 0.15 M NaCl the binding of protamine to DNA is cooperative.

To probe further the cooperativity of binding in 0.15 M NaCl, supernatant fractions from the precipitation curve obtained under these ionic conditions were dialyzed against 1.4×10^{-3} M NaCl, 10^{-4} M citrate, pH 7.0, and melted. A single transition corresponding to naked DNA was observed in all cases (data not shown).

3.2. Thermal denaturation of squid protamine-DNA complexes

It is known that protamines increase the thermal stability of DNA [11,13–15]. We have studied the thermal denaturation of reconstituted complexes of DNA and squid protamine.

Fig. 3 shows the derivatives of the melting profiles for rapid and slow complexes. In both cases the melting curves are clearly bimodal. The relative amount of the second transition increases in parallel with the (+/−) ratio. No significant turbidity changes were detected during the melting process, which indicates that melting of DNA does

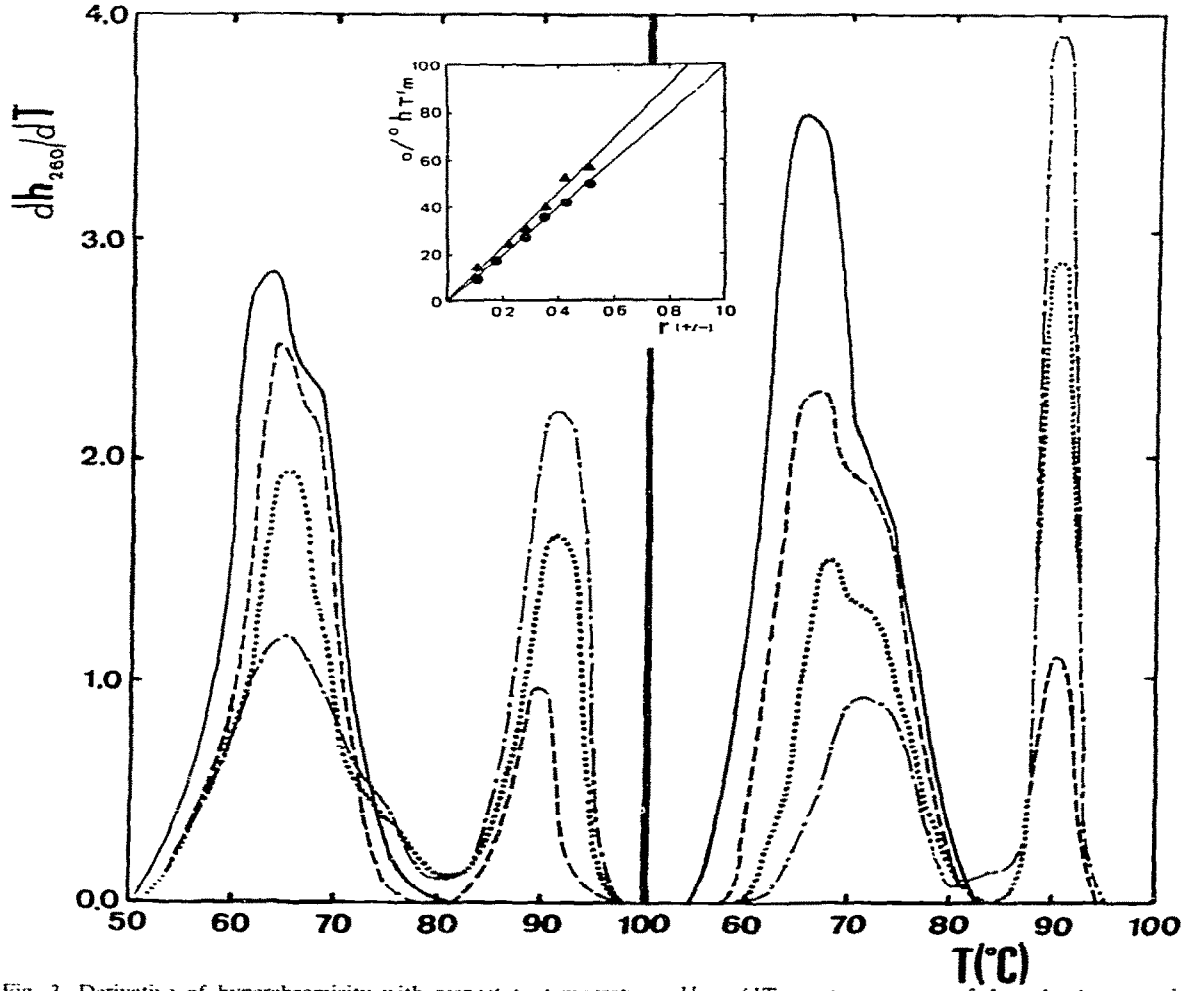

Fig. 3. Derivative of hyperchromicity with respect to temperature, dh_{260}/dT, vs. temperature of *L. vulgaris* protamine-DNA complexes formed by rapid binding (left) and slow binding (right). The input ratios (+/−) were: (———) 0, (- - - - - -) 0.15, (· · · · · ·) 0.35 and (- · - · -) 0.82. (+/−) = Arg/nucleotide ratio. The inset shows the percentage of hyperchromicity corresponding to the DNA protected regions ($\%h_{T_m}$) against the input ratio. (●) Rapid binding, (▲) slow binding. Thermal denaturation in 1.4×10^{-3} M NaCl, 10^{-4} M sodium citrate, pH 7.0.

not change the size of the molecular aggregates.

The melting temperature of the first transition, T_{m1}, corresponds to DNA not complexed with protein. In the rapid complexes its value does not vary significantly with the (+/−) ratio. The increase in the melting temperature of the first transition in the slow complexes with the (+/−) ratio may be related to the length of the DNA stretches that melt cooperatively. The binding of protamine to DNA, which induces a large difference in thermal stability from that of neighboring DNA regions, will reduce cooperativity of melting, particularly at low ionic strength [16]. Thus, shortening of the effective length of free DNA by the distribution of protamine-bound regions could give rise to an increase in the T_m of free DNA. The melting temperature of the second transition, T'_m, is not affected either by the method of preparation or the protamine/DNA ratio. In all complexes the value of T'_m is 90–91°C. The second transition is very sharp, in particular in the slow complexes. The melting temperatures are summarized in table 2.

If we consider the percentage of hyperchromicity associated with the second transition as equivalent to the fraction of DNA protected by protein against thermal denaturation, then the plot of these percentages against the (+/−) ratio gives the stoichiometry of protein binding [14]. In the rapid complexes a ratio (+/−) is obtained by extrapolation to 100% protection, whereas in the slow complexes 100% protection is obtained with a (+/−) ratio of 0.86. The different stoichiometry shown by rapid and slow complexes may result from looped or extended configurations of the nonbasic protamine residues as shown in fig. 6A.

In spite of the differences observed in the complexes prepared by slow and rapid methods, all of them show a similar overall behavior upon denaturation. All complexes display a biphasic profile. The first transition band can be attributed to the melting of free DNA and the second transition band to the DNA protected by protein. Both steps are clearly separated in the melting process. The high cooperativity of the second transition indicates that the complex is structurally very homogeneous, particularly in the case of the slow complexes.

3.3. Thermal melting of protein φ1- and φ3-DNA complexes

Melting of complexes of DNA with proteins φ1 and φ3 shows two major transitions associated with the melting of naked and fully protected DNA regions, as in melting of nucleoprotamines, but, at intermediate temperatures, other transitions are also present (fig. 4). The main transition band for the melting of protected DNA regions has a melting temperature of 90–94°C, depending on the protein and the method of preparation. These values are similar to those found for fish [11,13–15] and squid nucleoprotamines. Slow complexes show one (φ1 complexes) or two (φ3 complexes) intermediate transition bands. Both φ1 and φ3 rapid complexes show two intermediate transition bands. In these complexes the intermediate transitions amount only to a small percentage of the melting of the protected regions in the rapid complexes. In the slow complexes they represent approx. 50% of the hyperchomicity associated with the melting of protected regions. Thus, it appears that the slow method increases the contribution to the melting of the intermediate transitions. The coexistence of different degrees of DNA stabilization has also been observed in reconstituted complexes of DNA with basic peptides [16], statistical copolymers of L-lysine and L-valine [17] and single histones [18]. Different degrees of electrostatic shielding by protein basic residues; unshielded phosphates covered by the protein binding site, or destabilization of the DNA by protein nonbasic residues, could contribute to the presence of several thermal phase transitions in the protected DNA regions. The melting temperatures are summarized in table 2.

The stoichiometry of binding was calculated as already described for squid protamine complexes, although in this case all transitions bands associated with the different degrees of DNA protection were considered in the calculation of the fraction of DNA protected against thermal denaturation. As regards protein φ1, values of 1 and 0.9 are found for the (+/−) ratio in the protected regions in rapid and slow complexes, respectively. In the case of protein φ3, the calculated values for rapid and slow complexes are 1.5 and 0.9, respec-

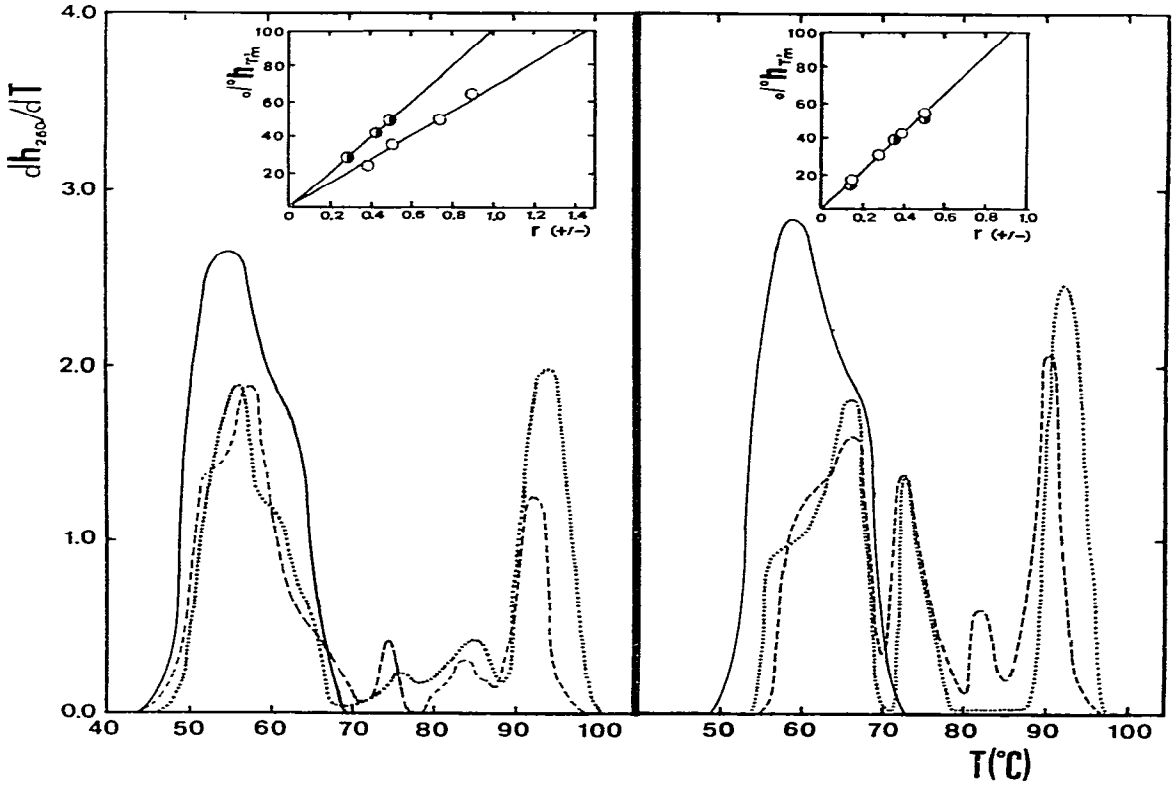

Fig. 4. Derivative of hyperchromicity with respect to temperature. dh_{260}/dT, vs. temperature of *M. edulis* φ1-DNA complexes (· · · · · ·) and *M. edulis* φ3-DNA complexes (- - - - - -) prepared by rapid binding (left) and slow binding (right). The input ratio, (+/−) = Arg + Lys/nucleotide, was in every case equal to 0.4. The insets show the percentage of hyperchromicity corresponding to the DNA protected regions ($\%h_{T_m}$) against the input ratio. (◑) *M. edulis* φ1, (○) *M. edulis* φ3. Thermal denaturation in 10^{-3} M sodium cacodylate, 10^{-4} M EDTA, pH 7.4.

tively. Thus, the values of the stoichiometry obtained from thermal denaturation agree well with the results from the precipitation curves.

3.4. X-ray diffraction

The study of fibers of complexes of DNA with basic proteins by X-ray diffraction allows a direct determination of some of the conformational parameters of the DNA in the complexes.

Fibers pulled from reconstituted complexes of DNA and squid protamine always give crystalline diagrams [2,19] which indicates that the DNA double helix is not irregularly distorted upon interaction with protamine. The alignment along the fiber axis of the DNA molecules in the complexes is strongly dependent on the method of preparation. Complexes prepared by mixing at low ionic strength always give disoriented diagrams which indicates that DNA molecules are not free to align themselves along the pulling direction. The orientation is improved when complexes are prepared by gradient dialysis. The orientation can still be improved by pulling the fibers at high ionic strength to make the binding of protamine molecules to DNA reversible (fig. 5) [2]. The inefficacy

Table 2

Melting temperatures for reconstituted nucleoproteins

β is the stoichiometry.

	L. vulgaris protamine		*M. edulis* ϕ1		*M. edulis* ϕ3	
	Rapid binding	Slow binding	Rapid binding	Slow binding	Rapid binding	Slow binding
T'_m I	66	65–70	56	66	58	66
T'_m I	–	–	76	72	74	72
T'_m II	–	–	85	–	83	82
T'_m III	89–92	90.5	94	92	92	90
β	1	0.86	1.04	0.9	1.47	0.9

of pulling fibers at low ionic strength in orienting the DNA molecules suggests the existence of protamine cross-links in the complex.

The analysis of fiber data by several authors shows that in nucleoprotamine DNA is in the B form and packed in a simple hexagonal lattice.

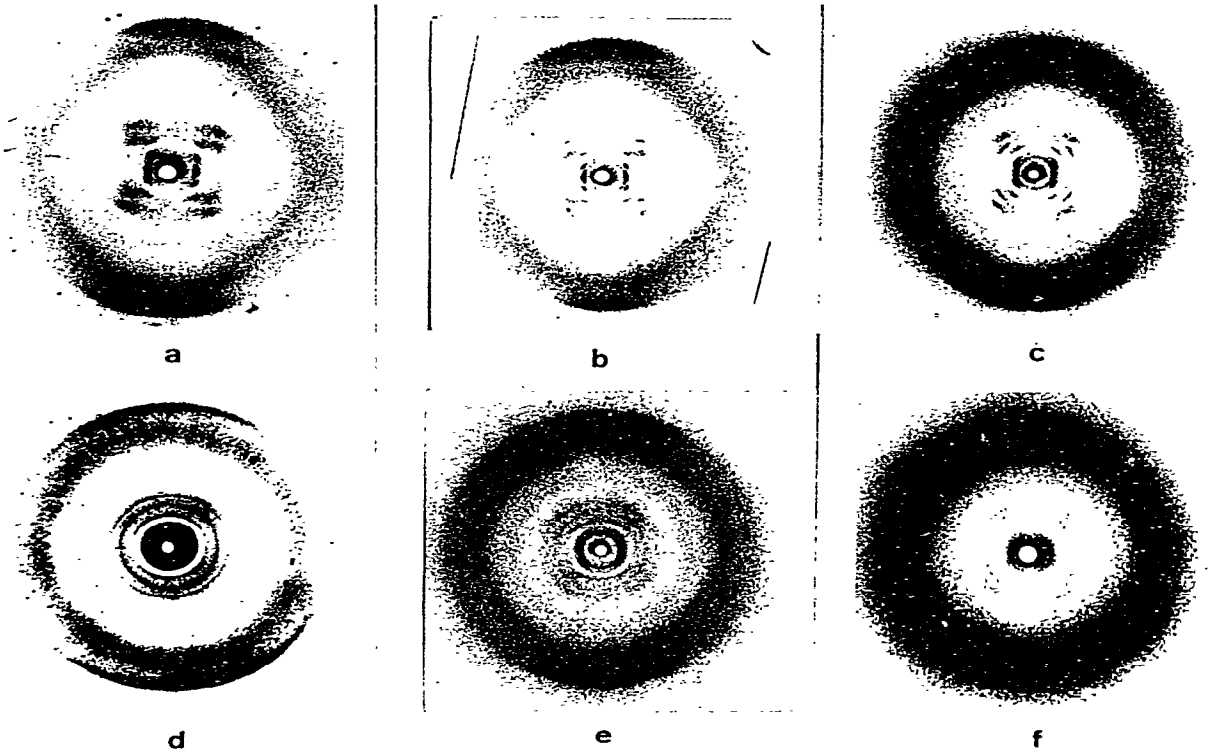

Fig. 5. X-ray diffraction diagrams at 92% relative humidity from complexes of *L. vulgaris* protamine (a, d), *M. edulis* ϕ1 (b, c) and *M. edulis* ϕ3 (c, f) with DNA. The upper set of diagrams (a–c) is from complexes prepared by rapid binding; the lower set (d–f) is from compiexes prepared by slow binding. The lattice parameters for the diagrams shown in this figure are summarized in table 3.

Packing disorder is present. The B form is stabilized at low humidities [2,20–22,26,27].

Protein φ1 behaves basically as squid protamine. Diagrams obtained from fibers prepared by gradient dialysis and pulled at high ionic strength are more oriented than those obtained by direct mixing at low ionic strength. As in the case of squid and salmonid protamines, DNA is in the B form and the first layer line is more intense than in the alkaline salts of DNA. The B form is also stabilized against the transition towards the A form at low humidities. The unit cell is hexagonal with a single molecule per unit cell.

In contrast to protamines and protein φ1, it is not possible to obtain protamine-like crystalline diagrams from protein φ3-DNA complexes unless the rapid method is used. When the slow method is used the diagrams show low crystallinity, which indicates that the structure of DNA is irregularly distorted. The diagrams from complexes prepared by rapid binding are protamine-like, in particular they show a strong first layer line. In contrast, those obtained from slow complexes show a weak first layer line.

Table 3 summarizes the lattice parameters obtaines by X-ray diffraction.

The chemical analysis of the fibers used for X-ray diffraction gave a (+/−) ratio of 0.8–1.0 for squid protamine and φ1 fibers. In the case of protein φ3 the ratios were approx. 1.5 and approx. 1 for rapid and slow complexes, respectively. These values are in agreement with the results of precipitation and melting experiments.

4. Discussion

We have studied several properties of proteins φ1 and φ3 from mussel sperm nuclei and compared them to those of the typical protamine from squid sperm nuclei. Protein φ1 appears to be very close to typical protamines. Protein φ3 shares some properties with typical protamines, but it also includes complex features which do not allow its inclusion in the same group of typical protamines.

The precipitation curves and the X-ray diagrams obtained with protein φ1 are similar to those obtained with typical protamines. Either type of protein shows noncooperative binding to DNA at low salt and cooperative binding in 0.15 M NaCl. Under both ionic conditions and for either protein, complete precipitation of the DNA occurs at a (+/−) ratio of approx. 1.

The X-ray diagrams from φ1-DNA complexes are similar to those obtained from nucleoprotamines. They always show good crystallinity. The orientation depends on the reversibility of the binding of the protein molecules to DNA during the process of pulling the fibers, as in nucleoprotamines. The DNA is in the B form and the lattice is hexagonal with a single molecule per unit cell. As in nucleoprotamines, the transition of the DNA to the A form at low humidities is blocked. The first layer line is intense; this fact being currently interpreted as reflecting the location of the protein relative to the DNA [20–22].

The melting of φ1-DNA complexes shows intermediate transitions. The percentage of hyperchro-

Table 3

Unit-cell parameters of reconstituted nucleoprotamines

Protein		100 spacing (nm)	0010 spacing (nm)	Ref.
L. vulgaris protamine	rapid binding	2.17	0.334	2
	slow binding	2.20	0.333	2
M. edulis φ1	rapid binding	2.25	0.336	4
	slow binding	2.20	0.335	4
M. edulis φ3	rapid binding	2.65	0.331	4
	slow binding	2.42	0.339	4

micity associated with these transitions is higher in complexes prepared by the slow method than in those prepared by the rapid method. Therefore, the rapid method accentuates the protamine character of the binding of protein ϕ1. Diffraction diagrams and precipitation curves do not distinguish protein ϕ1 from typical protamines. Only the presence of intermediate transitions in the melting of its complexes with DNA justifies its inclusion in the group of intermediate protamines. Thus, as far as the techniques employed are concerned, protein ϕ1 appears to be very close to typical protamines.

Protein ϕ3 differs more from typical protamines than does protein ϕ1. First, protein ϕ3 is very inefficient in precipitating DNA at low ionic strength. The results from melting and precipitation curves indicate that when the binding takes place under conditions of low ionic strength a rather large number of protein basic residues cannot reach the DNA phosphates. Alternative explanations can be given for this fact: (i) the conformation of bound protein may be such as to hinder sterically the binding of some of its basic residues to the DNA phosphates; (ii) under conditions of low ionic strength isolated basic residues may bind weakly to the DNA phosphates and, thus, be included in loops (fig. 6); (iii) a low cooperativity binding constant could give rise to gaps smaller than the DNA-binding site, yet ϕ3 molecules with a flexible binding site could bind to those sites leaving out some of its basic residues. However, at physiological ionic strength, values of 0.9–1.0 for the (+/−) ratio in protein-bound

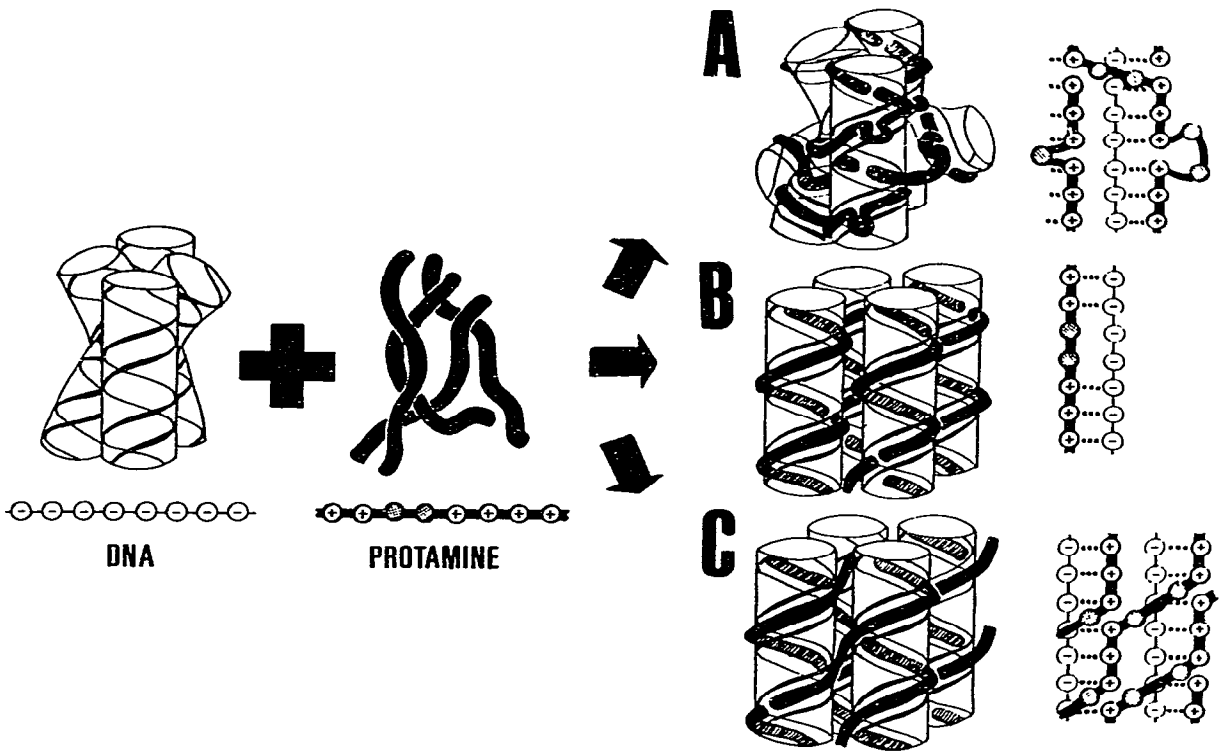

Fig. 6. Schematic representation based on Wilkins' model of some aspects of nucleoprotamine structure that could be heterogeneous. (A) Reconstituted nucleoprotamine by rapid binding, (B) reconstituted nucleoprotamine by slow binding, (C) native nucleoprotamine.

regions are found as in typical nucleoprotamines, which indicates that under these ionic conditions, the mode of binding of protein φ3 has changed.

The X-ray diagrams from φ3-DNA complexes show important differences as a function of the method of preparation. X-ray diagrams obtained from fibers prepared by rapid binding are protamine-like. As in nucleoprotamines, the first layer line is strong and the second and third layer lines are crystalline. In contrast, the diagrams obtained from complexes prepared by the slow method are not protamine-like. They show a very weak first layer line, which indicates that the electron density across the DNA grooves differs from that in nucleoprotamines. Another peculiar feature of the slow complexes is that the structure of the DNA is irregularly distorted as reflected in the poor crystallinity of the second and third layer lines (fig. 5).

The protamine-like diagrams obtained when φ3-DNA complexes are prepared by rapid binding at low ionic strength may be the result of a faulty reconstitution that occurs when the binding is dominated by electrostatic forces. At higher ionic strength hydrophobic forces may also play an important role as discussed by Mandel and Fasman [17]. Moreover, in the slow method the protein binds to the DNA under conditions of reversibility. Since the binding of protein molecules is virtually irreversible at low salt, in the rapid method of preparation the complex may remain kinetically blocked in conformations different from that of lowest energy [23,24].

Fig. 6 shows schematically the types of structural heterogeneity that could explain the influence of the reconstitution methods on the properties of the complexes. For convenience they are ilustrated according to Wilkins' model [20–22] where the protamine chain adopts an extended configuration and the basic residues stretch out towards the sides of the sugar/phosphate backbones that limit the narrow groove. The aspects of the binding that could be heterogeneous are; (i) the total or partial saturation of the DNA phosphates giving rise to looped (fig. 6A) or extended (fig. 6B) configurations of the nonbasic residues; (ii) the distribution of protamine cross-links between DNA molecules; (iii) in the case of protein φ3-DNA complexes with values of the stoichiometry higher than 1, isolated basic residues could also be included in loops.

The structural heterogeneity of the protein component in reconstituted complexes has also been shown by Warrant and Kim [25] by single-crystal X-ray diffraction of protamine-tRNA complexes prepared by diffusion of protamine into tRNA crystals. Only a small portion of the protamine molecule located in the major groove of the T stem of the tRNA is visible in the electron-density map. Since single-crystal studies can only reveal the ordered portion of the protamine molecule, this observation indicates that protamine molecules are only partially ordered in the crystal lattice.

How regular the arrrangement of protamine molecules complexed with DNA in sperm heads is remains an open question.

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